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Studies on *Bacillus pyocyaneus*.

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SINCE Gessard (1882) first isolated *B. pyocyaneus* in pure culture, an extensive literature on this organism has accumulated. The investigations have been done almost exclusively along four lines; *i. e.*, its proteolytic power, pathogenicity, antagonistic action for certain bacteria, and the pigments produced by it. Jordan (1899 *a*, 1903) has made the most notable contributions in the latter field, and has also shown that its hemolytic property is due to the alkali produced. The work of Wassermann (1896) on toxin production would apparently need further investigation. Pribram and Pauley (1915) reported upon the agglutination reactions within the fluorescent group of bacteria. They noted a strong antigenic relationship between *B. pyocyaneus* and *B. fluorescens liquefaciens*. While its ability to produce proteolytic and reducing enzymes has been extensively studied, very little has been done in regard to its ability to ferment carbohydrates, higher alcohols, etc. Flügge (1896) mentions its ability to ferment glycerol. Jordan (1899 *a*) (1899 *b*) states that *B. pyocyaneus* does not produce gas in dextrose broth fermentation tubes. He noted fermentation of dextrose by other fluorescent organisms. Fuller (1899), on the other hand, reported gas production, while McFarland (1919) states that *B. pyocyaneus* does not ferment carbohydrates. De Bord (1923) has recently reported that the rate of production of amino nitrogen in cultures of *B. coli, Ps. pyocyanea, B. subtilis, Cl. botulinum,* and *Cl. sporogenes* in peptone media is accelerated when glucose is present. He further states that *Ps. pyocyaneus* destroys glucose without markedly increasing the hydrogen-ion concentration and concludes that pH determination is fallible as an index of sugar destruction.

Clawson and Young (1913), working in this laboratory, reported cyanide production by *B. pyocyaneus*. Patty (1921) continued this work by studying the optimum conditions for cyanide production and comparing the quantities produced by nine strains. The present paper is the third in this series. The number of strains has been increased to twenty-two. The experimental work herein embodied has been carried out with the hope of throwing light upon the following questions:

1. How many, if any, of the twenty-two strains ferment dextrose and lactose as judged by growing them in 1 per cent sugar meat infusion broth and Hiss serum water media, using litmus as an indicator?

2. Would quantitative sugar determinations on cultures grown in 1 per cent dextrose meat infusion broth indicate sugar utilization by any of the strains giving negative results with litmus as an indicator?

3. What would be the hydrogen-ion concentration developing in such cultures?

4. What would be the reactions of a reasonable number of the strains in mannite, saccharose, arabinose, dulcite, xylose, salicin and rhamnose?

5. What would be the results of comparative studies on their ability to blacken lead acetate agar, produce indol in Dunham's peptone solution and reduce nitrates to nitrites?

6. How do the additional thirteen strains and also a culture of *B. fluorescens liquefaciens* compare as to cyanide production?

7. How do the twenty-two strains compare antigenically as evidenced by the agglutination reaction.

MEDIA AND METHODS.

1. Plain meat extract broth and fresh meat infusion broth, using Witte's peptone, and made according to standard methods, were used as bases for the sugar broths and agar media employed. The customary method for preparing Hiss serum water media was followed. A 1 per cent concentration of the fermentable substance was added. The broth was tubed in 10 cc. amounts in hard glass test tubes and in 150 cc. amounts in pyrex glass Erlenmeyer flasks. One-tenth cc. of a 24-hour meat infusion broth culture was used to inoculate the tubes and 0.5 cc. for inoculating the flasks. Pigment production and reduction of litmus were factors which prevented satisfactory readings to be made for more than a few days except where an adsorbing substance was added.

2. The hydrogen-ion concentrations were determined by the colorimetric method as suggested by Clark and Lubs. To prevent interference of pigment, one gram of sterile animal charcoal was added to each 150 cc. of media in the flasks. This apparently adsorbed the pigment. Control flasks of sterile media were used. The cultures were carried along in triplicate for two-week periods. This work was rechecked, thus giving six sets of determinations for each organism. The buffer solutions used were checked by means of a potentiometer.

3. Quantitative sugar determinations were done on a number of strains using Benedict's method.

4. Lead acetate agar was prepared as recommended by Jordan (1917).

5. Indol was tested for by the vanillin reaction (Nelson, 1916).

6. Cyanide determinations were made as previously described by Patty (1921).

7. For agglutination work the macroscopic method was used.

SOURCES OF ORGANISMS USED.

The history of the organisms studied is given in Table I.

TABLE I.

Strain No.	*Source.*
68	American Museum.
174	University of Chicago and Rush Medical College.
175	University of Chicago and Rush Medical College.
176	University of Chicago and Rush Medical College.
177	University of Chicago and Rush Medical College.
178	Gizzard strain.
179	University of Michigan.
180	University of Chicago and Rush Medical College.
184	Stool of influenza patient at Lawrence, Kan.
250	American Museum strain No. 150.
251	American Museum strain No. 151.
252	American Museum strain No. 152.
253	American Museum strain No. 156.
254	American Museum strain No. 206.
255	Dawson strain, Bureau of Animal Industry. American Museum strain No. 102.
256	American Museum strain No. 129 (The Jackson strain).
257	Bullock strain, Lister Institute No. 42 A.
258	Goat strain, Lister Institute No. 254.
259	Fildes strain, Lister Institute No. 150 B.
260	From the stool of an influenza patient at Lawrence, Kan.
261	From the stool of a typhoid fever patient at Lawrence, Kan.
262	From the stool of a typhoid fever patient at Lawrence, Kan.

RESULTS.

The results of the fermentation reactions, growth in lead acetate agar, Dunham's peptone solution and nitrate broth are shown in Table II. This table also contains the results of quantitative cyanide determinations for each strain grown in synthetic broth as described by Patty (1921).

TABLE II.

Strain No.	Results of carbohydrate fermentation as determined by indicators.									Blackening of lead aceate agar.	Indol.	No. 2.	HCN mg. per 100 cc.
	Dex.	Lact.	Man.	Sacch.	Arab.	Dul.	Xyl.	Sal.	Rham.				
68	—	—	—	+	—	—	—	—	+	—	+	—	.05925
174	—	—	—	+	—	—	+	+	—	—	+	—	.....
175	+	—	—	+	—	—	—	+	+	+	+	—	.....
176	+	—	—	+	—	—	—	—	+	+	+	—	.....
177	+	—	—	+	—	—	—	+	+	+	+	—	.....
178	—	—	—	+	—	—	+	+	+	—	+	—	.00953
179	+	—	—	+	—	—	—	+	—	+	+	—	.....
180	—	—	—	+	—	—	—	+	+	—	+	—	.....
184	+	—	—	+	—	—	—	—	—	—	+	—	.....
250	—	—	.....	.....	.....	.....	.....	.....	.....	+	+	—	.02706
251	+	—	.....	.....	.....	.....	.....	.....	.....	+	+	—	.00766
252	+	—	.....	.....	.....	.....	.....	.....	.....	—	+	—	.02041
253	—	—	.....	.....	.....	.....	.....	.....	.....	+	+	—	.01069
254	—	—	.....	.....	.....	.....	.....	.....	.....	+	+	—	.02413
255	+	—	.....	.....	.....	.....	.....	.....	.....	+	+	—	.08010
256	?	—	.....	.....	.....	.....	.....	.....	.....	+	+	—	.01910
257	+	—	.....	.....	.....	.....	.....	.....	.....	—	+	—	.01832
258	+	—	.....	.....	.....	.....	.....	.....	.....	—	+	—	.04330
259	+	+	.....	.....	.....	.....	.....	.....	.....	+	+	+	.01688
260	+	—	—	+	—	—	—	+	+	—	+	—	.02695
?261	?	—	.....	.....	.....	.....	.....	.....	.....	—	+	—	.04950
262	+	—	.....	.....	.....	.....	.....	.....	.....	—	+	—	.01550
B. fluorescens liquefaciens													.05126

Table III shows a number of hydrogen-ion concentrations for each organism grown in fresh meat infusion dextrose broth having an initial pH of 7.0 and containing 1 gram of animal charcoal to adsorb the pigment.

TABLE III.

STRAIN NO.	Hydrogen-ion concentration in 1% meat infusion dextrose broth expressed in pH.								
	Days.								
	0	1	2	3	4	5	6	7	14
68	7.0	7.0	7.0	7.2	7.6	7.6	7.8	8.0	8.0
174	.....	7.0	7.0	7.2	7.2	7.8	7.8	8.0	8.0
175	.....	7.0	7.0	6.8	6.6	6.6	6.6	6.6	6.6
176	.....	7.0	6.8	6.8	6.8	6.8	6.8	6.8	6.8
177	.....	6.6	6.8	7.0	7.0	7.4	7.6	7.6	7.8
178	.....	7.0	7.0	7.4	7.4	7.4	.....	7.8	7.8
179	.....	7.0	7.0	7.0	7.0	6.8	6.6	6.4	6.4
180	.....	7.2	7.2	7.4	7.4	7.6	7.6	7.8	7.8
184	.....	7.0	6.6	6.6	6.6	6.6	.....	6.6	6.0
250	.....	7.0	7.0	7.6	7.6	7.6	.....	8.0	8.4
251	.....	7.0	.....	6.6	6.6	7.0	.....	7.4	7.6
252	.....	6.4	.....	6.2	6.8	7.0	.....	7.0	7.6
253	.....	7.0	.....	7.0	6.6	7.0	.....	7.2	8.0
254	.....	7.0	.....	7.4	7.4	7.4	.....	7.8	8.0
255	.....	6.4	.....	6.4	6.8	7.0	.....	7.2	7.2
256	.....	7.0	.....	6.6	6.6	6.8	.....	7.0	7.6
257	.....	6.6	.....	6.8	6.8	7.0	.....	7.0	7.6
258	.....	7.0	.....	7.0	5.6	5.8	.....	6.0	6.8
259	.....	5.8	.....	5.4	5.4	5.4	.....	5.2	5.0
260	.....	7.0	.....	6.8	6.4	6.4	.....	6.2	7.0
261	.....	6.8	.....	7.2	7.4	7.6	.....	7.8	8.0
262	.....	6.6	.....	6.6	6.6	6.6	.....	7.0	7.8

Table IV gives the results of studies on seven strains of *B. pyocyaneus* to ascertain their ability to utilize dextrose. Three of these strains had given negative reactions in dextrose broth using litmus as an indicator, while the other four strains had shown an acid reaction. The reactions to litmus and the hydrogen-ion concentration developing in a neutral dextrose charcoal broth are herein compared with the percentage of sugar utilized daily for four days.

TABLE IV.

STRAIN NO.	Reaction in litmus dextrose meat infusion broth.	PH in 1% meat infusion dextrose charcoal broth.						Dextrose concentration of broth expressed in grams per 100 cc.				
		Days.						Days.				
		0	1	3	5	7	14	0	1	2	3	4
68	—	7.0	7.0	7.0	7.6	8.0	8.0	1.14	1.10	0.92	0.67	0.67
178	—	7.0	7.0	7.4	7.4	7.8	7.8	1.14	1.14	1.12	1.06	1.06
250	—	7.0	7.0	7.6	7.6	8.0	8.0	1.14	1.14	1.05	0.72	0.51
184	+	7.0	6.6	6.6	6.6	6.6	6.0	1.14	0.97	0.77	0.72	0.69
252	+	7.0	6.4	6.2	7.0	7.0	7.6	1.14	1.13	1.02	0.59	0.54
259	+	7.0	5.8	5.4	5.4	5.2	5.0	1.14	1.03	0.91	0.83	0.71
260	+	7.0	7.0	6.8	6.4	6.4	7.0	1.14	0.88	0.78	0.69	0.61

DISCUSSION OF TABLES II, III, IV.

An inspection of Table II indicates that fifteen out of the twenty-two strains ferment dextrose, and one strain, the Fildes strain, obtained from the Lister Institute, fermented both dextrose and lactose. These results are confirmed by the hydrogen-ion concentrations tabulated in Table III. The data in Table IV suggest that the apparently negative strains did utilize dextrose, but that the alkali produced kept pace with acid production, and finally alkali production predominated. This is of interest, since it apparently throws more light upon the principle of the interference of fermentation with proteolysis and is in keeping with what one would expect from the standpoint of physiology. The principle that the presence of a fermentable carbohydrate in a culture would in general result in proteolysis being held in abeyance until the concentration of carbohydrate had been reduced to an exceedingly small amount has been studied extensively by Kendall (1921). Peckham (1897) announced this phenomenon for the colon group, and mentions that Pere had previously called attention to it. Our results suggest that, given the proper hydrogen-ion concentration, both processes may be initiated.

Subsequent results depend largely upon the development of an optimum hydrogen-ion concentration for either fermentation or proteolysis. Apparently, sugar was utilized at pH 7.6 which is not an incompatible reaction for proteolysis. These results, as well as those of DeBord (1923), and Downs (1924) of this laboratory, also emphasize the limitations of the indicator methods in deciding upon the ability of an organism to ferment a carbohydrate. Mannite, arabinose, and dulcite were apparently negative, while saccharose was uniformly fermented by ten of the strains studied. The results in xylose, salicin and rhamnose were variable.

There was great uniformity in the production of indol, but only half of the twenty-two strains blackened lead acetate agar. Only one reduced nitrates to nitrites. Jordan (1899*a*) noted that his cultures of *B. pyocyaneus* produced indol, but that the fluorescent organisms gave variable results. He also noted that nitrates were strongly reduced by the Rush strain of *B. pyocyaneus*, slightly reduced by *B. pyocyaneus alpha*, and not reduced by five other strains. Tanner (1917) studied ninety-seven strains of fluorescent bacteria and one strain of *B. pyocyaneus* to ascertain whether any or all of these organisms produced hydrogen sulfid. He found that the majority of the fluorescent bacteria produced it, but that the one culture of *B. pyocyaneus* failed to do so. Sasaki and Otsuka (1912) noted that one strain of *B. pyocyaneus* did not produce hydrogen sulfid.

In regard to cyanide production, our results were quantitatively less than reported by Patty (1921). This was probably due to environmental factors in the culture media, as the same methods were used and repeated checks made. The production of cyanide by *B. fluorescens liquefaciens* (American Museum strain) is another characteristic in common with *B. pyocyaneus*.

ANTIGENIC RELATIONSHIP.

For this work adult rabbits were immunized against strains 68, 174, 175, 176, 177, 178, 179, 180, 184 and 260 respectively. Two rabbits were inoculated for each strain. Each animal received ten subcutaneous inoculations at weekly intervals and several small doses of live cultures intravenously. The results were disappointing. None of the titres were high, but varied from zero to 1-5000. The results of cross agglutination were irregular.

SUMMARY AND CONCLUSIONS.

The results of the comparative study of the carbohydrate and protein metabolism and of the antigenic relationship of twenty-two strains of *Bacillus pyocyaneus* maye be summarized as follows:

1. Practically all strains of *B. pyocyaneus* have the ability to utilize dextrose although alkaline products may obscure this phenomenon. They were consistently negative in mannite, arabinose and dulcite, and positive in saccharose.

2. Our results as well as those of DeBord (1923) and Downs (1924) emphasize the limitations of hydrogen-ion determinations as an indication of sugar utilization.

3. The Fildes strain was the only one of the twenty-two strains studied that showed acid production in litmus lactose broth. It also produced the maximum hydrogen-ion concentration in fresh meat infusion dextrose broth, reaching a pH of 5.0 in two weeks. Six of the twenty-two strains showed an acid reaction after two weeks. The remaining sixteen strains developed pH values ranging from 7.0 to 8.0 at the end of fourteen days. The initial reaction was pH 7.0. These results might not hold if the initial hydrogen-ion concentration were *decidedly* different.

4. Animal charcoal added to dextrose broth apparently adsorbs the pigments.

5. All strains produced indol.

6. Half of the twenty-two strains blackened lead acetate.

7. Only one of the twenty-two strains reduced nitrates.

8. All strains produced HCN, as did also a strain of *B. fluorescens liquefaciens*.

9. Efforts to produce high titred immune sera were not very successful; a titer of 1-5000 was the maximum obtained. Cross agglutination experiments gave irregular results.

BIBLIOGRAPHY.

1. CLAWSON, B. J., and YOUNG, C. C. 1913. Jour. Biol. Chem., 15, 3.
2. DEBORD, GEO. G. 1923. Jour. Bact., 8, 1.
3. DOWNS, CORNELIA M. 1926. University of Kans. Science Bul.
4. FLUGGE. Die Microörganisms. 1896.
5. FULLER, GEO. W., and JOHNSON, GEO. A. 1899. Jour. Exp. Med., 4, 5 and 6.
6. GESSARD. La pyocyanine. Thése, Paris, 1882. (Reference from Jordan's General Bacteriology. 1924. W. B. Saunders Co.)
7. JORDAN, E. O. 1899 *a*. Jour. Exp. Med., 4, 5 and 6.
8. JORDAN, E. O. 1899 *b*. The Botanical Gazette, 27, 1.
9. JORDAN, E. O. 1903. Jour. Med. Res., 10, 1.
10. JORDAN, E. O. 1917. Jour. Inf. Dis., 21, 6.
11. KENDALL, A. I. Bacteriology, General, Pathological and Intestinal. 1921. Lea and Febriger.
12. MCFARLAND, JOSEPH. Pathogenic Bacteria and Protozoa. 1919. W. B. Saunders Co.
13. NELSON, V. E. 1916. Jour. Biol. Chem., 29.
14. PATTY, F. ARTHUR. 1921. Jour. Inf. Dis., 29, 1.
15. PECKHAM, ADELAIDE WARD. 1897. Jour. Exp. Med., 2, p. 549.
16. PRIBRAM, E., and PAULAY, E. 1915. Centralbl. f. Bakt., etc. 1, Abt. Orig. 76, 5.
17. SASAKI, TAKOAKI and OTSUKA, ICHIRO. 1912. Biochem. Zeitschr. 39, p. 208.
18. TANNER, FRED W. 1917. Jour. Bact., 2, 5.
19. WASSERMANN, A. 1896. Zeitschr. f. Hyg., 22.